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# **SILICA-IMMOBILIZED ENZYME REACTORS; APPLICATION TO CHOLINESTERASE- INHIBITION STUDIES**

**Heather R. Luckarift, Glenn R. Johnson  
Air Force Research Laboratory  
Tyndall AFB, FL 32403-5323**

**Jim C. Spain  
School of Civil & Environmental Engineering  
Georgia Institute of Technology  
Atlanta, GA 30332**

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139 Barnes Drive, Suite 2  
Tyndall AFB, FL 32403-5323**

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/signed/  
\_\_\_\_\_  
GLENN A. JOHNSON  
Work Unit Manager

/signed/  
\_\_\_\_\_  
SANDRA R. MEEKER  
Chief, Airbase Sciences Branch

/signed/  
\_\_\_\_\_  
WENDELL D. BANKS  
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## Silica-immobilized enzyme reactors; application to cholinesterase-inhibition studies

Heather R. Luckarift<sup>a,\*</sup>, Glenn R. Johnson<sup>a</sup>, Jim C. Spain<sup>b</sup>

<sup>a</sup> Air Force Research Laboratory, Airbase Technologies Division, 139 Barnes Drive, Suite #2, Tyndall Air Force Base, FL 32403, USA

<sup>b</sup> Georgia Institute of Technology, School of Civil and Environmental Engineering, 311 Ferst Drive, Atlanta, GA 30332, USA

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### Abstract

A rapid and economical method is reported for the preparation of an immobilized enzyme reactor (IMER) using silica-encapsulated equine butyrylcholinesterase (BuChE) as a model system. Peptide-mediated silica formation was used to encapsulate BuChE, directly immobilizing the enzyme within a commercial pre-packed column. The silica/enzyme nanocomposites form and attach simultaneously to the metal affinity column via a histidine-tag on the silica-precipitating peptide. BuChE–IMER columns were integrated to a liquid chromatography system and used as a rapid and reproducible screening method for determining the potency of cholinesterase inhibitors. The IMER preparation method reported herein produces an inert silica-encapsulation matrix with advantages over alternative systems, including ease of preparation, high immobilization efficiency (70–100%) and complete retention of activity during continuous use.

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**Keywords:** Immobilized enzyme reactor; Butyrylcholinesterase; Silica-encapsulation; Cholinesterase inhibitors

### 1. Introduction

Immobilization of proteins to solid supports is advantageous for a wide variety of biosensing, bioprocessing and affinity chromatography applications. Immobilized enzyme reactors (IMERs) have found application in catalysis and have also been used with a wide variety of receptor proteins for substrate interaction and inhibition studies [1–7]. The main advantages of immobilized enzyme systems are stability and reusability. In addition, IMERs facilitate continuous on-line analysis, significantly minimizing cost and analysis time. Immobilization of enzymes for IMER applications has been demonstrated using a variety of chemical and physical techniques. Chemical immobilization generally involves enzyme attachment to a matrix via cross-linking or covalent bonding. Physical methods include adsorption of biomolecules to a porous support or ion exchange matrix, or entrapment within an insoluble gel matrix. Several previously reported IMER configurations use silica or monolithic materials as a support matrix for enzyme immobilization

but such systems are often handicapped by poor enzyme loading capacities. Many methods of immobilization and entrapment also cause significant structural deformation of the enzyme, leading to reduction in activity. Significant optimization of the immobilization method is often required and factors such as stability may be sacrificed in favor of increased loading capacity [3–6].

Butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) are crucial to transmission of nerve impulses in mammals and have received increasing attention due to their potential roles in disorders of the central nervous system, such as Alzheimer's disease and Down's Syndrome [8]. BuChE is of pharmacological and toxicological importance due to its ability to hydrolyze ester-containing drugs and scavenge cholinesterase inhibitors, including organophosphate nerve agents [9]. Inhibition of cholinesterase provides a mechanism for acetylcholine replacement, which has proven to be an effective therapy in treating the cognitive and functional symptoms of Alzheimer's disease [10,11]. IMERs have found increasing application to acetylcholinesterase inhibition studies by employing immobilized acetylcholinesterase or horseradish peroxidase within packed columns [4,12–14]. The previously reported systems however, have specific

\* Corresponding author. Tel.: +1 850 283 6034; fax: +1 850 283 6090.  
E-mail address: [hluckarift@gtcom.net](mailto:hluckarift@gtcom.net) (H.R. Luckarift).

drawbacks such as low loading capacity and long preparation times.

We recently reported a biomimetic silicification reaction that provides a biocompatible and simple method for enzyme immobilization resulting in a stable, heterogeneous catalyst with enhanced mechanical stability and high loading capacity [15,16]. The silicification reaction mixture consists of hydrolyzed tetramethyl orthosilicate (TMOS) and a silica-condensing synthetic peptide (R5). The R5 peptide is the repeat unit of a silaffin protein previously identified from the diatom *Cylindrotheca fusiformis*. In the diatom, silaffins catalyze the precipitation of silica, to form the organism's exoskeleton. The R5 peptide mimics the silica precipitation *in vitro* and forms a network of fused spherical silica nanoparticles (average diameter of 500 nm) [17].

The stability of silica-immobilized enzymes provided an opportunity to explore continuous flow-through reaction systems. Silica-entrapped BuChE was initially investigated in two flow-through systems: (1) a fluidized-bed system and (2) a packed-bed system. The fluidized-bed system proved suitable for continuous operation and retained conversion efficiency for over 1000 column volumes, but the use of the column was limited by the need for upwards flow-through the column, to prevent packing. In the packed-bed system the conversion rate decreased over time; the immobilized enzyme was not inactivated during the continuous flow but rather the overall retention time decreased, due to packing and eventual channeling of the silica particles [15]. The mechanical stability of the silica-immobilized enzyme indicated that it was applicable to flow through applications but the configuration of the apparatus required optimization. In order to avoid these limitations, the aim of the present study was to determine whether IMERs could be prepared using silica-encapsulation *in situ* via his-tag attachment of the silica-immobilized enzyme to metal ion affinity resin. Immobilization of silica to surfaces has recently been reported by attachment of silicatein proteins to a gold surface, using histidine-binding to nickel via a nitrilotriacetic acid chelator [18]. An alternate method involves deposition of silica by electrochemical dip pen nanolithography patterning of histidine-tagged peptides [19]. Simultaneous encapsulation and attachment of an active biomolecule to the surface of a flow-through device however, has not been previously reported. The silica immobilization method reported herein provides a novel method for rapid and highly efficient enzyme encapsulation and is applicable to the preparation of a wide variety of immobilized biomolecules.

## 2. Experimental

### 2.1. Materials

Butyrylcholinesterase (E.C.3.1.1.8; from Equine Serum,  $\approx 50\%$  protein and activity of 1200 Units/mg protein) was purchased from Sigma–Aldrich (St. Louis, MO). Cholinesterase specific phosphate buffer was used throughout (0.1N NaOH, 0.1 M  $\text{KH}_2\text{PO}_4$ , pH 8) unless otherwise stated [15]. All other chemicals were of analytical grade and obtained from Sigma–

Aldrich. The synthetic peptides; R5 (SSKKS<sub>6</sub>SYSGSKGSKRRIL), C-terminus (His)<sub>6</sub>-tag R5: (SSKKS<sub>6</sub>SYSGSKGSKRRILHHHHHHH-COOH), N-terminus (His)<sub>6</sub>-tag R5: (H<sub>2</sub>N-HHHHHHSSKKS<sub>6</sub>SYSGSKGSKRRIL) were from New England peptides (Gardner, MA).

### 2.2. Enzyme analysis

The activity of BuChE was determined by the rate of butyrylthiocholine iodide (BuCh-I) hydrolysis in potassium phosphate buffer (25 mM, pH 7.0) containing  $\text{MgSO}_4$  (10 mM) and Ellman's reagent (1.26  $\mu\text{M}$ ); the reaction produces a yellow anion that can be detected by spectroscopy, where  $\epsilon = 13,600 \text{ m}^{-1} \text{ cm}^{-1}$  at 412 nm [12–14,20,21]. A calibration curve of the thiocholine product complex was generated by incubating fixed concentrations of BuCh-I with BuChE until the reaction reached completion (assumed to be 100% conversion). The absorbance was measured at 412 nm and correlated with the product extinction coefficient [20]. Protein concentration was determined by using a bicinchonic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as standard.

### 2.3. His-tag immobilization to agarose beads

A stock solution of the R5 peptide (or (His)<sub>6</sub>-R5) (100 mg/ml) was prepared in deionized water. Silicic acid was prepared by hydrolyzing TMOS (final concentration 1 M) in hydrochloric acid (1 mM). Chelating sepharose fast flow metal ion affinity chromatography media was charged with cobalt ions (1 M  $\text{CoCl}_2$ ) according to the manufacturer's instructions (GE Healthcare/Amersham Biosciences, Piscataway, NJ). The silicification mixture consisted of BuChE stock solution (80  $\mu\text{l}$  of 100 U/ml), hydrolyzed TMOS (10  $\mu\text{l}$ ) and R5 peptide stock (10  $\mu\text{l}$  of 100 mg/ml). The ratio of (His)<sub>6</sub>-R5 peptide and R5 peptide was varied to determine loading capacity but the final peptide concentration of the mixture was maintained at 10 mg/ml throughout. The mixture was left for 30 min to allow the silicification reaction to proceed and then washed with five volumes of buffer.

### 2.4. IMER preparation

#### 2.4.1. C-His<sub>6</sub>-BuChE-IMER and N-His<sub>6</sub>-BuChE-IMER

BuChE-IMERs were prepared using HiTrap Chelating HP columns (dimensions: 1.6 cm  $\times$  2.5 cm; 5 ml volume) charged with cobalt ions (1 M  $\text{CoCl}_2$ ) according to the manufacturer's instructions (GE Healthcare/Amersham Biosciences, Piscataway, NJ). The (His)<sub>6</sub>-R5 peptide (500  $\mu\text{l}$  of 10 mg/ml) was loaded onto the column and washed according to the manufacturer's instructions. The silicification mixture, consisting of BuChE stock solution (1.6 ml of 100 U/ml), hydrolyzed TMOS (0.2 ml) and R5 peptide stock (0.2 ml) was mixed and added to the column. The column was left for 30 min to allow the silicification reaction to proceed and then washed with five column volumes of buffer.

#### 2.4.2. Si-BuChE-IMER

The Si-BuChE-IMER was prepared as above with the exception of the His<sub>6</sub>-R5 peptide.

#### 2.4.3. Soluble-BuChE-IMER

The soluble-BuChE-IMER was prepared by loading soluble enzyme (1.6 ml of BuChE stock solution (100 U/ml)) directly onto the column. The column was left for 30 min before washing with five column volumes of buffer.

After immobilization, the BuChE activity and protein concentration in the eluate and resultant wash fractions were measured to determine the immobilization efficiency. For stability studies, buffer was passed through the columns continuously at a fixed rate (1 ml/min). At regular intervals, the residual enzyme activity on the columns was determined.

The morphology of the silica nanoparticles was characterized by scanning electron microscopy (ICBR Electron Microscopy Core Lab, University of Florida).

### 2.5. Chromatography conditions

For activity and inhibition studies, the IMERs were attached to an Agilent 1100 series liquid chromatography system. Phosphate buffer (25 mM, pH 7.0) was used as the mobile phase at a flow rate of 1 ml/min, unless otherwise stated, and the eluate was monitored using a diode-array detector (412 nm). BuCh-I was injected onto the IMER columns (concentration range: 10  $\mu$ M–250 mM, 20  $\mu$ l injections in triplicate) and the peak area of the product was correlated to concentration against a calibration curve. Blank control samples (containing no inhibitor) were injected at regular intervals to monitor the reproducibility and stability of the column. Michaelis–Menten plots were generated of activity (mmoles product/min) at a range of substrate concentrations and specific activity ( $V_{\max}$ ) values were calculated using GraphPad Prism software (v 3.02). For inhibition experiments a stock solution of inhibitor (100 mM) was prepared in ethanol and diluted into a solution of BuCh-I (200 mM) to give a range of inhibitor concentrations (10  $\mu$ M–10 mM). The degree of inhibition was determined according to the formula  $I(\%) = (I_i - I_f)/I_i \times 100$ , where  $I_i$  is the initial steady state absorbance of the substrate, and  $I_f$  corresponds to the final activity of the enzyme in the presence of inhibitor. Inhibition curves (percentage activity inhibition versus log [inhibitor]) were plotted and the IC<sub>50</sub> values extrapolated using GraphPad Prism software (v 3.02).

## 3. Results and discussion

### 3.1. Butyrylcholinesterase immobilization

The effect of additional histidine residues upon the silicification activity was determined using the R5 peptide with six histidine residues (his-tag) attached at either the carboxyl (C)-terminus or amino (N)-terminus. Both (His)<sub>6</sub>-tagged peptides catalyzed the precipitation of silica at a rate comparable to the native R5 peptide indicating that the addition of histidines does not affect the precipitation activity of the peptide (data

not shown). The R5 peptide typically produces silica nanoparticles with an average size of  $\sim$ 500 nm [15–17]. SEM analysis revealed that the C-(His)<sub>6</sub>-R5 peptide catalyzed the formation of silica particles with a size range of approximately 150–700 nm and an average size of  $\sim$ 500 nm. The silica particles formed by the N-(His)<sub>6</sub>-R5 were slightly larger, with a size range of 700–1200 nm and an average size of  $\sim$ 800 nm (data not shown).

The suitability of metal ion affinity chromatography media for enzyme immobilization was determined initially using a slurry of the column packing material in batch experiments in order to optimize the enzyme immobilization conditions. The maximum loading capacity of the silica nanoparticles formed by precipitation with (His)<sub>6</sub>-R5 was approximately 20 Units BuChE per milliliter packing media. The enzyme loading could be increased to approximately 30 Units BuChE per milliliter of packing media by using a mixture of one part (His)<sub>6</sub>-R5:four parts R5. Encapsulation with (His)<sub>6</sub>-R5 alone limits enzyme immobilization to the surface of the agarose beads. The presence of non-tagged peptide, however, increases the formation of an interconnected matrix of silica nanospheres (Fig. 1), therefore greatly increasing the surface area for encapsulation. We previously determined that the silicification reaction yields approximately 1.2 mg of silica from a 100  $\mu$ l reaction mixture [15]. The calculated capacity for enzyme loading in the silica nanospheres using the optimized reaction conditions (above) is  $\sim$ 22.2 mg enzyme/g silica (2.2%, w/w).

### 3.2. Butyrylcholinesterase-IMER preparation

The scheme for immobilizing BuChE into a packed column is shown in Fig. 2. A pre-packed metal ion affinity chromatography column charged with cobalt ions selectively retains proteins (or peptides) with histidine or other complex-forming amino acid residues, exposed on the surface of the protein. Therefore a His<sub>6</sub>-homologue of the R5 peptide selectively binds to the cobalt ions. When the silicification mixture is applied to the column, silica precipitation occurs and integrates with the peptide already bound to the column, resulting in the concurrent immobilization of the enzyme. Analysis of the packing within the column by SEM confirmed the presence of silica nanospheres attached to the surface of the agarose beads (Fig. 1 c and d).

Four columns were prepared comprising: (1) soluble BuChE (soluble-BuChE-IMER); (2) BuChE immobilized in silica (Si-BuChE-IMER); (3) BuChE immobilized in silica, with N-terminal His<sub>6</sub>-peptide (N-His<sub>6</sub>-BuChE-IMER); and (4) BuChE immobilized in silica, with C-terminal His<sub>6</sub>-peptide (C-His<sub>6</sub>-BuChE-IMER). The amount of protein retained during immobilization was determined for each IMER. In the case of the silica-immobilized IMERs, it is difficult to determine what proportion of BuChE was bound to the column because unbound peptide would also be detected in the eluate. The columns that contain the silica however, retained much more total protein (>90%) than the soluble-BuChE-IMER (Table 1). Residual BuChE activity in the eluate and wash fractions was negligible in all cases (less than 1%—data not shown). The enzyme loading for the silica-immobilized columns was approximately 30 Units BuChE per ml packing, in agreement with the maximum immo-



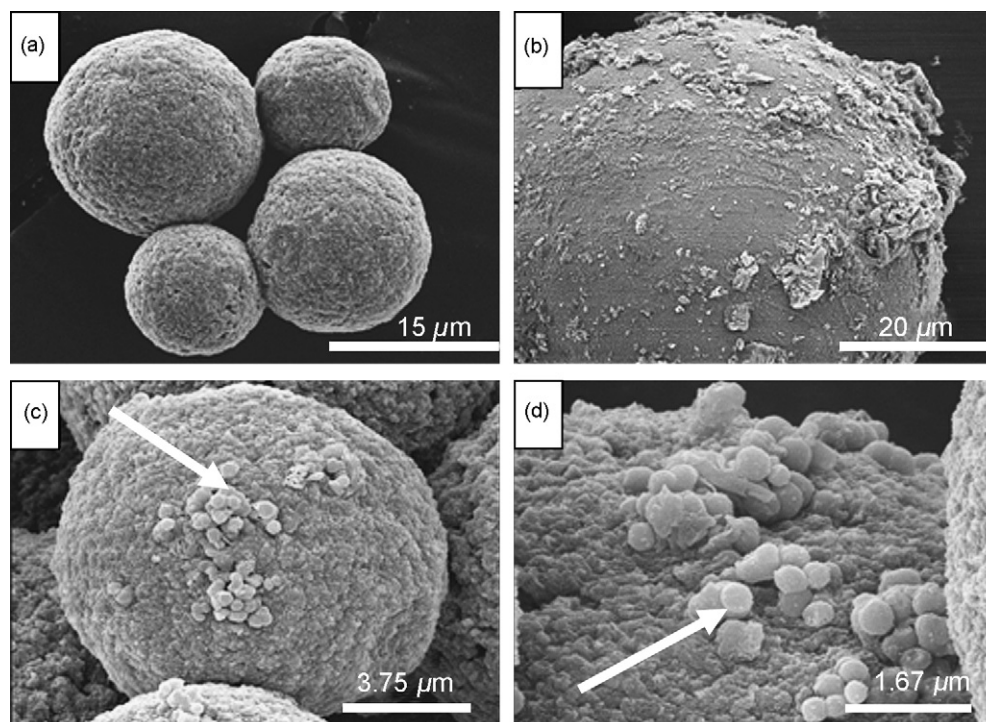


Fig. 1. SEM micrographs of silica nanoparticles attached to agarose beads SEM analysis of agarose beads (a) and immobilized BuChE attached to agarose beads. Using silica nanoparticles formed from N-(His)<sub>6</sub>-R5 peptide only (b) or from a mixture of N-(His)<sub>6</sub>-R5 peptide and R5 peptide (c and d).

bilization capacity previously obtained during optimization in loose media.

The silica-immobilized IMERs exhibited high substrate conversion efficiency (~60%) irrespective of the presence or absence of the his-tag. Despite the high immobilization efficiency in the absence of a his-tag however, the Si-BuChE-IMER lost activity over time and was attributed to the gradual elution of silica particles from the column during continuous flow. The

physical attachment of the silica particles via the his-tag resulted in stable IMER preparations, which during continuous flow conditions, demonstrated reproducible conversion of BuCh-I for both the C-His<sub>6</sub>-BuChE-IMER and N-His<sub>6</sub>-BuChE-IMER with no significant loss in enzyme activity or conversion efficiency (Fig. 3). The His<sub>6</sub>-BuChE-IMERS were stable over a period of 200 column volumes of continuous flow. The silica immobilization provided greater stability and retention of enzyme activity

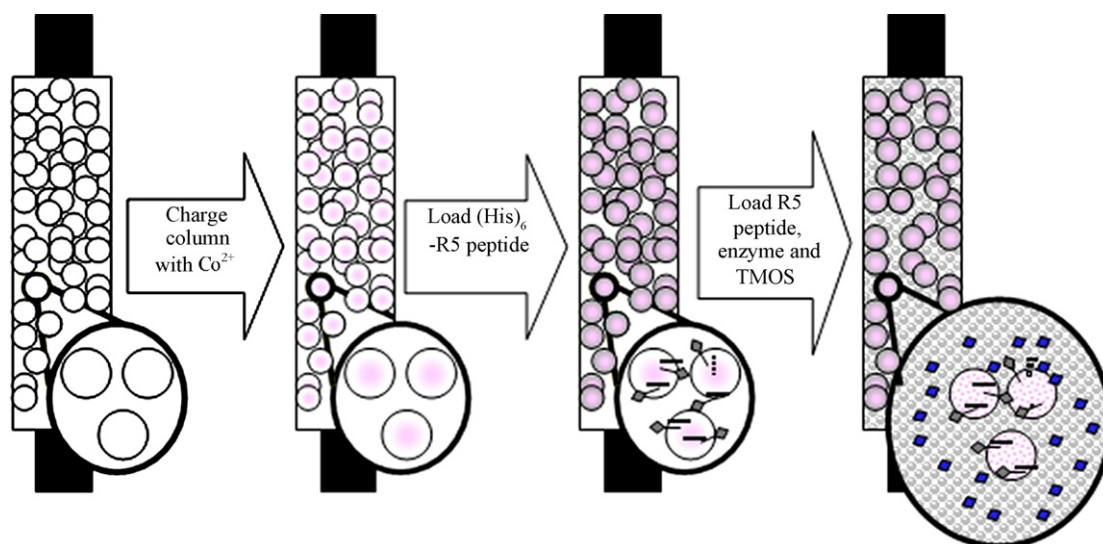


Fig. 2. Scheme for enzyme immobilization in silica nanospheres attached by affinity binding to cobalt-coated resin. Key: agarose beads (○); Co<sup>2+</sup> coated agarose beads (●); his-tagged peptide (—); enzyme (■); silica nanospheres (■).

Table 1  
Immobilization efficiency and kinetic parameters of BuChE–IMERs

		Soluble-BuChE–IMER	Si-BuChE–IMER	N-His <sub>6</sub> -BuChE–IMER	C-His <sub>6</sub> -BuChE–IMER
Column contents	BuChE	✓	✓	✓	✓
	R5 peptide	×	✓	✓	✓
	His <sub>6</sub> -R5 peptide	×	×	✓	✓
Protein retained (%) <sup>a</sup>		47.7	92.55	98.69	98.95
Immobilized units (Units) <sup>b</sup>		~93	~158	~160	~131
Immobilization efficiency (%)		58.1	98.7	100	70.6
Enzyme activity ( $V_{\max}$ ) (μmoles/min)		18.68 ± 0.42	31.58 ± 0.65	32.48 ± 0.92	26.28 ± 1.16

<sup>a</sup> ([Protein]<sub>in</sub> – [Protein]<sub>out</sub>).

<sup>b</sup> ([Units]<sub>in</sub> – [Units]<sub>out</sub>).

than the soluble-BuChE–IMER. The initial conversion activity of the soluble-BuChE–IMER was significantly lower (~48%) and it lost activity rapidly.

### 3.3. Determination of kinetic parameters of BuChE–IMERs

N-His<sub>6</sub>-BuChE–IMER and C-His<sub>6</sub>-BuChE–IMER columns connected to an LC system exhibited stable performance at a wide range of flow rates from 0.5 to 3 ml/min. Multiple injections of substrate through the columns by means of an auto sampler system provided rapid analysis and demonstrated reproducible conversion efficiency. The percentage conversion of BuCh-I and the product retention time decreased with increasing flow rate as expected due to the reduction in residence time. The column pressure remained stable and below 70 Bar at the range of flow rates tested (data not shown). A flow rate of 1 ml/min was chosen as an optimum balance between high product conversion and low retention time. Under the optimum flow conditions the chromatographic retention time was approximately 5 min and analysis of an injected sample was completed in less than 10 min.

The retention of BuChE activity by each of the IMERs indicated that BuChE was retained on the stationary phase. The relative activity and rate of reaction of BuChE immobilized within the IMERs was determined using Michaelis–Menten plots to determine specific activity ( $V_{\max}$ ) (Fig. 4). True kinetic parameters cannot be defined using this fixed-bed system, because the initial reaction rates cannot be determined due to the residence

time in the columns which results in complete conversion at low substrate concentrations. The specific activity of the IMERs can, however, be used to compare specific activity between like systems and provides an estimate of immobilization efficiency. For each system, the hydrolysis of BuCh-I followed conventional Michaelis–Menten kinetics and saturating substrate concentration was in excess of 100 mM (Fig. 4).

Because  $V_{\max}$  is directly proportional to enzyme concentration, the units of immobilized enzyme can be correlated to  $V_{\max}$  as described previously [14] (Table 1). The immobilization efficiency was highest for the IMERs that involved silica-immobilization of the enzyme. However, significant non-specific binding of the free enzyme was observed, which is intriguing considering the low percentage (~1%) of histidine residues in BuChE. Recent reports suggest a non-competitive interaction between BuChE and metal ions such as Ni<sup>2+</sup> and Co<sup>2+</sup>, which might contribute to the non-specific binding observed in this study [22]. The soluble enzyme, however, was not retained within the soluble-BuChE–IMER during continuous flow operation and some variability in the data obtained from the soluble-BuChE–IMER was recognized and is attributed to the loss in enzyme activity during continuous analysis.

### 3.4. Inhibition of BuChE–IMERs

The silica-based IMERs showed stable and reproducible conversion of BuCh-I during continuous operation, providing a system that is suitable for a number of applications that would not be feasible with soluble enzyme. The hydrolysis of BuCh-I by cholinesterases is decreased by the presence

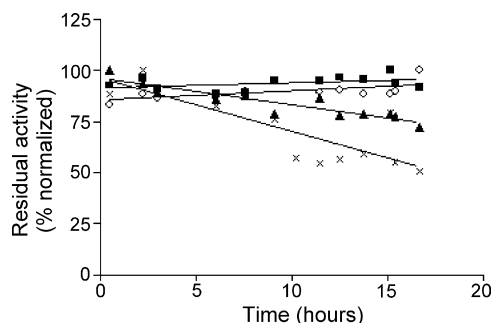


Fig. 3. Stability of BuChE–IMERs during continuous operation. (×) Soluble-BuChE–IMER; (▲) Si-BuChE–IMER; (○) C-His<sub>6</sub>-BuChE–IMER; (■) N-His<sub>6</sub>-BuChE–IMER. Conversion activity (%) normalized to initial rate. Based on concentration of product (μM) from conversion of 100 μM BuCh-I at a flow rate of 1 ml/min.

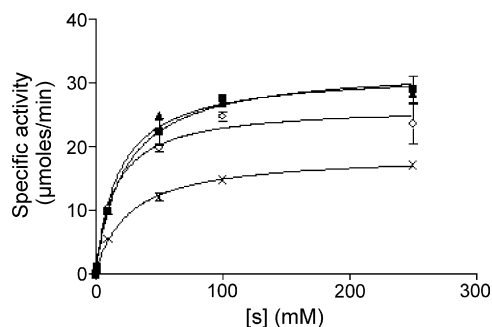


Fig. 4. Michaelis–Menten plots for BuChE–IMERs. (×) Soluble-BuChE–IMER; (▲) Si-BuChE–IMER; (○) C-His<sub>6</sub>-BuChE–IMER; (■) N-His<sub>6</sub>-BuChE–IMER. Values are mean and SD of triplicate experiments.



Table 2  
Effect of cholinesterase inhibitors on BuChE activity in IMERs

Inhibitor	N-His <sub>6</sub> -BuChE-IMER		C-His <sub>6</sub> -BuChE-IMER	
	IC <sub>50</sub> (mM)	K <sub>i</sub> (mM)	IC <sub>50</sub> (mM)	K <sub>i</sub> (mM)
Galantamine	0.65 ± 0.03	0.04	0.49 ± 0.04	0.05
Eserine	0.84 ± 0.03	0.05	0.88 ± 0.03	0.09
Tacrine	5.75 ± 0.15	0.40	4.37 ± 0.62	0.44
Edrophonium chloride	10.02 ± 2.69	0.70	11.69 ± 6.12	1.20

IC<sub>50</sub> values represent mean with SD of triplicate experiments.

of inhibitors and can therefore be measured in a continuous flow system for screening cholinesterase inhibitors and ranking of their inhibitory potencies. Four reversible inhibitors of BuChE were investigated; tacrine, eserine (physostigmine), galantamine and edrophonium chloride and were selected on the basis of their potency and mode of inhibition (Table 2). The BuChE-IMERs exhibited a concentration-dependent response to all of the cholinesterase inhibitors. In all cases, increasing inhibitor concentration resulted in concurrent and concentration-dependent reduction of BuCh-I hydrolysis. Galantamine was the most potent of the inhibitors tested. The IC<sub>50</sub> values were consistently higher than those determined *in vitro* but demonstrate feasibility of using IMERs to screen the preliminary inhibition characteristics of substrates. The inhibitor potency of eserine was approximately 5 times greater than observed for tacrine, in agreement with previous literature reports [23].

#### 4. Conclusion

The use of silica-encapsulation provides a facile immobilization technique that permits retention of enzyme activity and imparts mechanical properties that facilitate application to flow-through systems, such as IMERs. The IMERs can be used for the screening of specific enzyme inhibitors and the ranking of their inhibitory potencies; an extremely useful parameter in drug discovery. Butyrylthiocholine is not a physiological substrate for human brain butyrylcholinesterase but is used as a synthetic substrate for the enzyme. Therefore, inhibition constants derived using this method can only be representative of relative inhibitor potency. The primary advantage of the IMER system is integration into a liquid chromatography system, which facilitates application to high throughput screening. A wide variety of potential inhibitors can be screened by injecting the test compounds together with substrate and rapidly measuring inhibition kinetics.

A recent report describing immobilization of enzymes onto a microreactor surface using his-tag attachment was limited to commercially available or highly purified enzymes and resulted in very low enzyme loading [24]. The location of the his-tag on the silica-nucleating peptide rather than on the protein eliminates the need for recombinant modification of the protein of interest in order to use this method. The affinity binding of the silica peptide to the column resin provides a system that is durable under continuous use, with retention of activity at flow rates that are directly applicable to on-line chromatography appli-

cations. The IMERs were stable and reusable for analysis of over 250 injections, totaling more than 50 h of continuous use with no significant loss in activity. The automation of IMER analysis by integration into a LC system with an auto-sampler significantly reduces the time and work load required to analyze inhibitor potency, providing reliable and reproducible data within a short time period. The reusability of the IMERs also significantly reduces the amount of enzyme required for analysis.

The IMERs demonstrated in this study are presented as a model system applicable to a range of formats. The loading capacities achieved were sufficient for demonstrating the concept, but analysis of the silica-coated agarose indicated that we have only used a fraction of the surface of the agarose beads and further optimization of the approach will lead to dramatically higher loading capacities. Preliminary investigations indicate that a wide range of enzymes can be readily immobilized using the silica entrapment method [15,16] providing opportunities to create IMER systems of a variety of biomacromolecules with potentially interchangeable components. This bioencapsulation strategy therefore provides an economical and rapid route for synthesizing IMER systems with a number of advantages including; minimal preparation time, high immobilization efficiency and excellent stability. IMERs could be designed to contain an enzyme for biocatalysis or organic synthesis, for rapid screening in medical diagnostics and therapy or for developing IMER columns for affinity chromatography [1,2,25]. In addition, co-immobilization of multienzyme systems is also possible. Such systems can provide continuous cofactor recycling [26], or catalyze multistep processes. The method described is scalable dependent upon the application, for example, in a microfluidic format for biosensors or as large-scale IMERs for biosynthesis.

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